

Statistical Review and Evaluation (Carcinogenicity)

Date:

NDA No: 20-986

Applicant: Novo Nordisk Pharmaceuticals

Name of Drug: NovologTM, Insulin aspart (rNDA origin) injection

Document Reviewed: Insulin aspart Submission NDA 20-986, Response to Draft Package Insert of September 16, 1999, submitted November 5, 1999

X14 Toxicity to rats by repeated (twice daily) subcutaneous injection for 52 weeks, NN Study 940301 (T14), Volumes 1 and 3, dated November 15, 1996
Insulin X14 Exploratory 12 Months Toxicity in Rats, NN Study 930803 (T13), Dated December 20, 1994

Reviewing Pharmacologist: Indra Antonipillai, Ph.D., HFD-510

Reviewing Statistician: Karl K. Lin, Ph.D., HFD-715

1. Introduction

A draft physician's package insert for the drug product was provided by FDA in an approvable letter sent to the drug sponsor September 16, 1999. In the section on carcinogenicity, mutagenicity, impairment of fertility of the draft package insert, the following paragraph regarding the carcinogenicity of the drug was included:

"Standard two year carcinogenicity studies in animals have not been performed to evaluate the carcinogenic potential of NovologTM. In a 52-week _____ dosed subcutaneously with Novolog at 10, 50, and 200 U/kg/day

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The sponsor has included in this submission statistical analyses of data of mammary gland tumors from the toxicity study mentioned in the package insert and another similar toxicity study of the drug product to support its argument that this section of the FDA draft package insert should be replaced by the text the sponsor has proposed. Dr. Indra Antonipillai of HFD-510, reviewing pharmacologist of this NDA, has asked the Division of Biometrics II to conduct a statistical review of the sponsor's submitted statistical analyses.

2. Sponsor's Analysis and Results

The sponsor performed tests for trend, pairwise comparisons between the control group and each of the treated groups, and pairwise comparisons between human insulin and insulin apart (Novolog) at 200 U/kg/day using the methods described in Peto, et al. (1980)². The Peto methods of analysis of tumor data adjust mortality differences among treatment groups to eliminate or reduce possible biases caused by the differences.

Results of the sponsor's analysis of the mammary gland tumor data from Study 940301 (T14) are summarized in the following tables (compiled from Table 9-1 in the first document reviewed, and Table 4 in Appendix 16 from the second document reviewed).

All mammary gland tumors (Fibroadenoma, adenoma, and adenocarcinoma)

Treatment	Initial group size	One-year survivors	Animals bearing mammary tumors	P-value for one-tailed comparison against control	P-value for trend test	P-value for two-tailed comparison against HI
Control	32	29	7			
Iasp 10 U/kg/day	32	26	11	0.22		0.82
Iasp 50 U/kg/day	32	24	11	0.17	0.21	0.72
Iasp 200 U/kg/day	32	14	11	0.004	0.003	0.062
HI 200 U/kg/day	32	15	6	0.24		

² Peto, R., M.C. Pike, N.E. Day, R.G. Gray, P.N. Lee, S. Parish, J. Peto, S. Richards, and J. Wahrendorf (1980). "Guidelines for Simple, Sensitive Significance Tests for Carcinogenic Effects in Long-term Animal Experiments," In Long-term and Short-term Screening Assays for Carcinogens: An Critical Appraisal, World Health Organization.

Malignant mammary gland tumors (adenocarcinoma) only

Treatment	Initial group size	One-year survivors	Animals bearing mammary tumors	P-value for one-tailed comparison against control	P-value for trend test	P-value for two-tailed comparison against HI
Control	32	29	2			
Insulin 10 U/kg/day	32	26	4	0.11		0.41
Insulin 50 U/kg/day	32	24	2	0.48		0.82
Insulin 200 U/kg/day	32	14	4	0.090	0.090	0.17
HI 200 U/kg/day	32	15	1	0.54		

Benign mammary gland tumors (Fibroadenoma, and adenoma) only

Treatment	Initial group size	One-year survivors	Animals bearing mammary tumors	P-value for one-tailed comparison against control	P-value for trend test	P-value for two-tailed comparison against HI
Control	32	29	6			
Insulin 10 U/kg/day	32	26	9	0.27		0.95
Insulin 50 U/kg/day	32	24	10	0.15	0.18	0.71
Insulin 200 U/kg/day	32	14	8	0.051	0.039	0.42
HI 200 U/kg/day	32	15	5	0.25		

The sponsor presented the following findings from its analysis results:

- a. There was a statistical significant trend ($p=0.003$) among the control and Novolog (insulin aspart) treated groups in all mammary gland tumors (malignant and benign tumors combined). The trend

became not significant ($p=0.21$) when the group treated with Novolog at 200 U/kg/day was excluded in the test.

- b. There was a statistically significant increase ($p=0.004$) in all mammary gland tumors (malignant and benign tumors combined) in the pairwise comparison between the control group and the high dose group treated with Novolog at 200U/kg/day.
- c. There was a statistical significant trend ($p=0.039$) among the control and Novolog (insulin aspart) treated groups in benign mammary gland tumors (fibroadenoma and adenoma).
- d. There was a borderline significant increase ($p=0.051$) in benign mammary gland tumors (fibroadenoma and adenoma) in the pairwise comparison between the control group and the high dose group treated with Novolog at 200U/kg/day.
- e. There was no statistically significant increase in mammary gland tumor incidence (malignant and benign tumors separately or combined) in the low and medium dose groups treated with Novolog when they were compared with the control group.
- f. The pairwise comparisons in mammary gland tumors (malignant and benign separately or combined) between the group treated with human insulin at 200 U/kg/day with each of the Novolog treated groups were not statistically significant at 0.05 significance level.

The sponsor also performed a statistical analysis on the data from an additional 52-week toxicity study (Study 930803 (T13)). The data of the female rat study are summarized in the following table (compiled from the table on page 20 of the third document reviewed).

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Treatment	Novolog (200U/kg/day)	Human insulin (200U/kg/day)	Untreated
Animals per group at start	20	20	20
Preliminary deaths	3	8	0
Sacrificed for humane reasons	2	0	1
One year survivors	15	12	19
Animals having tissue examined by microscopy	18	17	20
Animals with adenoma(s) in mammary glands	7	8	4
Animals with adenocarcinoma(s) in mammary glands	4	3	1

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The statistical procedures used in this analysis were the same as those used in the analysis of data of study

T14. The sponsor reported the following findings from this analysis:

- a. There was a statistically significant increase in mammary gland benign tumor (adenoma) between the control group and the group treated with 200U/kg/day human insulin.
- b. There was no significant difference detected in mammary gland tumor incidence between the control group and the group treated with 200 U/kg/day Novolog.

- c. There was no significant difference ($p=0.52$) in mammary gland tumor incidence between the group treated with 200 U/kg/day Novolog and the group treated with 200 U/kg/day human insulin.

The sponsor performed the third analysis combining the data of studies T13 and T14. The following are the findings from this analysis:

- a. There was no statistically significant difference in mammary gland tumor incidence between between the group treated with 200 U/kg/day Novolog and the group treated with 200 U/kg/day human insulin.

3. Reviewer's Analysis

- a. The reviewer first evaluated the sponsor's statistical analyses reported in the three documents reviewed and had questions about how the analyses were performed. For example, how did the sponsor partition the one year study period into intervals in the survival-adjusted analyses? what were the numerators and denominators used in the calculations of the tumor proportions in the analyses? and if the contexts of observation of tumors were used in the selection of methods of analyses?

The reviewer contacted, with approval from HFD-510, Mr. Robert Fisher, Assistant Director of Regulatory Affairs of Novo Nordisk Pharmaceuticals in U.S. November 23, 1999 and requested for more detailed information about the company's statistical analyses. The above questions were presented to Mr. Fisher. Mr Fisher arranged a teleconference on November 29, 1999 for the reviewer to talk to Mr. Gert Neilsen (statistician of Novo Nordisk in Denmark), Mr. Lars Didericksen (toxicologist of Novo Nordisk in Denmark), Ms. Anita Osborne (regulatory affairs person of Novo

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Nordisk in Denmark), and Mr. Fisher. In the teleconference, the sponsor provided the reviewer with the details of the statistical analyses.

- b. The survival-adjusted procedures were carried out by partitioning the one year study period into 52 weekly intervals for fatal tumors and by partitioning the one year study period into two intervals before and after terminal sacrifice for incidental tumors. Data of fatal tumors were analyzed using the death-rate method, and data of incidental tumors using the prevalence method (both described in the paper by Peto, et al. (1980)). Both the exact version and the asymptotic version of the above methods were carried out. The results of the exact and the asymptotic analyses were similar.
- c. Based on the information presented in the sponsor's reports and in the teleconference, the reviewer feels that the statistical procedures the sponsor used in its statistical analyses described in Section 2 of this review and evaluation report are standard and valid.
- d. To determine whether the result a trend test or a pairwise comparison test is statistically significant, it is necessary to refer to predetermined levels of significance. In standard two-year, two-species, and two-sex carcinogenicity studies, the levels of significance listed in the following table are used. According to our studies (Lin and Rahman, 1998)³, the use of those decision rules will result in an overall false positive rate about 10% in a standard two-species-two-sex standard two-year study with 50 animals per sex/treatment group.

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Statistical Decision Rules for Controlling the Overall False Positive Rates Associated with Tests for Positive Trend or with Control-High Pairwise Comparisons in Tumor Incidences to

³ Lin, K.K., and M.A. Rahman, "Overall False Positive Rates in Tests for Linear Trend in Tumor Incidence in Animal Carcinogenicity Studies of New Drugs", Journal of Pharmaceutical Statistics, with discussion, 8(1), 1-22.

Around 10% in Carcinogenicity Studies of Pharmaceuticals.

	Tests for Positive Trend	Control-High Pairwise Comparisons
Standard Two-Year Studies with 2 Species and 2 Sexes	Common and rare tumors are tested at 0.005 and 0.025 significance levels, respectively.	Common and rare tumors are tested at 0.01 and 0.05 significance levels, respectively.
Alternative ICH Studies (One Two-Year Study in One Species and One Short or Medium-Term Study, 2 Sexes)	Common and rare tumors are tested at 0.01 and 0.05 significance levels, respectively.	[]

A tumor type is classified as rare if its spontaneous rate is 1% or less, and as common, otherwise.

- e. The above decision rules (levels of significance) are not appropriate for this study because (i) it is a one year study on female rats (one species and one sex) only, (ii) the numbers of animals per treatment groups was 32 in one study and 20 in the other study, and (iii) the mammary gland tumor in female rats was the only tumor type tested for evaluation of the carcinogenicity of the drug product. There were no multiple tests of other tumor types involved in the evaluation even though malignant and benign mammary gland tumors were tested separately and combined, and therefore there is no need to adjust the effect of multiple tests to control the overall false positive rate.

Without simulation studies, the appropriate levels of significance should be used in this study to determine the result of a test is statistically significant or not can not be determined. However, the reviewer feels that the usual significance level of 0.05 is appropriate for the trend tests and pairwise comparison tests in this review based on the above differences in nature between this study and the standard two-year study.

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- f. When the level of significance of 0.05 is used for the tests for trend and pairwise difference, this reviewer agrees with the following main conclusions presented in the sponsor's report submitted November 5, 1999 (first document reviewed):
- (i) Although the observed overall mammary gland tumor rates of the dose groups treated with 10 U/kg/day and 50 U/kg/day Novolog, 11/32 and 11/32, respectively, are higher than that of the control group, 7/32, they are not statistically different from the tumor rate of the control group in study T14. Due to the fact that we have only very limited sample data, and that different white noises and uncontrollable factors can cause fluctuations in observed values in a single study, conclusions should not be drawn based on observed values unless it is a clear-cut situation.
 - (ii) Also in study T14, Novolog induced mammary gland tumors in female Sprague-Dawley rats only at the dose level of 200 U/kg/day based on the significant trend test and the difference in control and high groups pairwise comparison in mammary gland tumor incidence.
 - (iii) In study T13, the difference in mammary gland tumor incidences between the control group and the high dose group treated with Novolog is not statistically significant.
 - (iv) In both studies T13 and T14, either analyzed separately or combined, the differences in mammary gland tumor rate between the group treated with 200 U/kg/day Novolog and the group treated with 200 U/kg/day are not statistically significant.
- g. It is noted that the survivals and mammary gland adenocarcinoma incidence rates (with data available for direct comparison) of the two groups treated with 200 U/kg/day Novolog and the two groups treated with 200 U/kg/day human insulin in studies T13 and T14 are significantly different. The survivals at 52 weeks and the adenocarcinoma tumor incidence rates of the two studies are given in the

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table below (on next page). The two groups in study T13 have significantly higher survivals than the two corresponding groups in study T14. The higher early deaths in the two groups in study T14 reduced the chance of developing more adenocarcinomas in the tested animals of the study. There is no data of benign mammary tumors (Fibroadenoma and adenoma) available in the sponsor's report for a direct comparison.

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The sponsor did not explain the possible factors that could cause the differences in survival and tumor incidence between the two studies. If the experimental conditions of the two studies were indeed different, then it would not be appropriate to combine the data of the two studies in the sponsor's third statistical analysis. However, this should not be an issue in the interpretation of the overall analysis results since the separate analyses of the two studies show the same results as the analysis using combining data.

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		Control	10 U/kg/day Novolog	50 U/kg/day Novolog	200 U/kg/day Novolog	200 U/kg/day human insulin
Study T13	Survivals	19/20 (95%)			15/20 (75%)	12/20 (60%)

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	Adenocar- cinoma tumor rates	1/20 (5%)			4/20 (20%)	3/20 (15%)
Study T14	Survivals	29/32 (91%)	26/32 (81%)	24/32 (75%)	14/32 (44%)	15/32 (47%)
	Adenocar- cinoma tumor rates	2/32 (6%)	4/32 (13%)	2/32 (6%)	4/32 (13%)	1/32 (3%)

- h. If the significant findings in the 200 U/kg/day group with Novolog can be considered as not clinically relevant based on the theory that the dose (200 times the therapeutic dose) is well above a possible threshold dose of a non-genotoxic compound^{4, 5} or other justifications, then the sponsor's proposed text for the carcinogenicity section of the physician's package insert seems reasonable.

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⁴ Ames, B.N., and L.S. Gold, "Too Many Rodent Carcinogens: Mitogenesis Increases Mutagenesis, Science, Vol. 249, pp970-971.

⁵ Cohen, S.M., and L.B. Ellwein "Cell Proliferation in Carcinogenesis", Science, Vol. 249, pp1007-1011.

Karl K. Lin, Ph.D.
Expert Mathematical Statistician
(Applications in Pharmacology and Toxicology)

Concur:

S. Edward Nevius, Ph.D.
Director, Division of Biometrics II

cc: Original/NDA 20-986 File
HFD-510/ IAntonipillai, RSteigerwalt
HFD-715/Chron
HFD-715/ENevius, Klin

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Addendum to the Pharmacologist's July 21, 99 review of NDA 20-986**A. LABELING**

Following issues were discussed further on September 10, 1999, after completion of the pharmacology review (dated July 21, 1999):

1. **Carcinogenicity Findings:** In a 52-week toxicity study in rats (QA certified study), all doses of the drug (X14 at 10, 50 and 200 U/kg/day) caused mammary gland tumors (benign + malignant) compared to vehicle controls (11, 11, 11 respectively vs 7 in controls $p=0.003-0.0039$), than human insulin (200 U/kg/day) compared to vehicle controls (6 vs 7 in controls $p=0.24$). This was analyzed by Peto's analysis, which shows that all doses significantly increased the mammary tumors, in the trend test. Also, slightly, but not statistically significant increases ($p=0.062$) in mammary gland tumors were observed between X14 and regular human insulin (both at 200 U/kg/day) in this study. The mammary gland tumor findings with X14 were above the historical control data.
2. **Pregnancy Category:** The pre- and post-implantation losses, and visceral/skeletal abnormalities were observed in both rats and rabbits with X14 (at approximately 32 and 3 times the human dose), and CFR indicates that this pregnancy category should be 'C'. Previous studies have shown that insulin given during pregnancy can induce structural changes in the offsprings in various species, this happens at doses as low as 1 U or less (Schardein J.L. in Chemically-induced birth defects, second edition, Marcel Dekker, Inc., New York and Basel). Furthermore, in pregnant rats, brief hypoglycemia with insulin treatment during organogenesis, can disrupt normal embryo development (J. Clin. Invest. 78: 643, 1986).

Based on these discussions, we have made additional labeling modifications (see attached label).

Carcinogenicity, Mutagenicity, Impairment of Fertility

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Standard 2-year carcinogenicity studies in animals have not been performed to evaluate the carcinogenic potential of _____ In a 52-

week study _____ dosed subcutaneously with _____ at 10, 50 and 200 U/kg/day (approximately 2, 8 and 32 times the _____ human subcutaneous dose of 1.0 U/kg/day, based on U/body surface area)

_____ was not genotoxic¹⁰⁴ in the following tests, Ames test, mouse lymphoma cell forward gene mutation test, human peripheral blood lymphocyte chromosome aberration test, in vivo micronucleus test in mice, and in ex vivo UDS test in rat liver hepatocytes. In fertility studies in male and female rats, at subcutaneous doses up to 200 U/kg/day (approximately 32 times the _____ human subcutaneous dose, based on U/body surface area), no direct adverse effects on male and female fertility, or general reproductive performance of animals was observed¹⁰⁵.

_____ "Pregnancy- Teratogenic Effects-Pregnancy Category C"

Subcutaneous reproduction and teratology studies have been performed with _____ and regular human insulin in rats and rabbits. In these studies _____ was given to female rats before mating, during mating and throughout pregnancy, and to rabbits during organogenesis. These effects of _____ did not _____ differ from those with subcutaneous regular human insulin _____ caused pre- and post-implantation losses, and visceral/skeletal abnormalities in rats at a dose of 200 U/kg/day (approximately 32-times the _____ human subcutaneous dose, based on U/body surface area), and in rabbits at a dose of 10 U/kg/day (approximately three times the _____ human subcutaneous dose, based on U/body surface area). No significant effects were observed in rats at a dose of 50 U/kg/day and rabbits at a dose of 3 U/kg/day. These doses are approximately 8 times _____ human subcutaneous dose,



Nursing- It is unknown whether _____ is excreted _____ in human milk.



The justification for the changes are as follows:

1. Carcinogenicity: The mammary gland tumor findings with X14 were greater than historical controls, and slightly greater than insulin. We believe this should be in the label to describe the findings. Slightly, but not statistically significant increases ($p=0.062$) in mammary gland tumors were observed between X14 and regular human insulin (at 32-times the human dose) in a 52-week toxicity study in rats (QA certified study). X14 also had a higher potential in promoting benign and combined (benign + malignant) mammary gland tumors compared to vehicle controls ($p=0.003-0.0039$), than human insulin compared to vehicle controls ($p=0.24$). However, X14 is not genotoxic. Therefore, under 'Carcinogenicity', the reviewer is suggesting the above text for labeling.
2. Pregnancy: Studies in rats were conducted up to 32-times the human dose, and in rabbits up to 3-times the human dose respectively, under _____ -Pregnancy', the reviewer is suggesting a change in title and the text for labeling. Since pre- and post-implantation losses, and visceral/skeletal abnormalities were observed in both rats and rabbits with X14 (at approximately 32 and 3 times the human dose), and even though these were similar to insulin, technically according to CFR, this makes it a pregnancy category 'C'. Insulin has no pregnancy category labeling, so there

is no clear comparison. Lys-pro (another analog) has category B in pregnancy labeling, since it had no findings (but it was only tested at 4 and 0.3 times the human dose in rats and rabbits respectively)

B. In pharmacologist's review of July 21, 99: Under overall summary and evaluation: other clinically relevant issues- the second 1-year toxicity study in rats.

The last sentence from this review on page 69 which states that

[] is deleted from the review. This is due to the fact that mammary tumors were observed in this study with all doses of the drug X14 (10, 50, and 200 U/kg/day).

C. Segment III reproductive Toxicity study

In the segment III toxicity studies in NDA 20-986 (submitted on 4/19/99, study # 940304), following is now added. The macroscopic examinations in segment III study showed that F1 offsprings had a slightly higher incidence of pups with minor kidney changes (mainly increased pelvic dilatation or pale coloration/enlarged kidneys) among litters derived from F0 females. This was found mainly in the right kidneys. The incidences were 1, 4, 7, 3 and 12 at 0, 10, 50, 200 U/kg/day of X14 and 200 U/kg/day with recombinant human insulin respectively. Therefore, incidences were higher with human insulin then with X14. Thus, minor kidney changes were noted with both, X14 and regular human insulin.

D. One-year toxicity studies in rats and dogs

An increase in alkaline phosphatase was observed in clinical studies by the medical reviewer, this was re-examined in animal toxicity studies. In 1 year toxicity study in rats, slight but not significant increases in alkaline phosphatase (ALP) were observed with X14 vs controls in week 51 (males 148-150 at 10-200 U/kg/day vs 136 mu/ml in controls, females 72-91 vs 74 mu/ml in controls). However, a significant increase in ALP was noted with recombinant human insulin at 200 U/kg/day in male rats in this study in week 51 (175* vs 136 mu/ml in controls, *p<0.05), but not in female rats (91 vs 74 mu/ml in controls).

In a 1 year dog toxicity study, slight increases in alkaline phosphatase (ALP) were also observed with X14 vs controls in week 52 (133 at 2 U/kg/day vs 99 mu/ml in controls). Similar slight increases in ALP were also noted with recombinant human insulin at 2 U/kg/day in dogs in this study in week 52 (112 vs 99 mu/ml in controls).

In summary, ALP tended to be slightly higher in X14 than in vehicle control groups, but it was also higher in animals treated with regular human insulin, and significance of this finding is unclear. In human studies ALP was significantly elevated in two studies.

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Indra Antonipillai
Pharmacologist, HFD-510

Team Leader concurs

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cc: NDA Arch
HFD510
HFD510/antonipillai/steigerwalt/koller/jrhee
Filename: _____

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Review completed: July 21, 1999

Sponsor : Novo Nordisk Pharmaceuticals Inc., 100 Overlook Center, Princeton, NJ.Date Submitted: September 15, 1998Date Received: September 16, 1998.Drug Class: Insulin Aspart (Insulin X-14, —————), L-Aspartic acid-insulin human
(Recombinant human insulin, DNA origin, β^{28} Asp-Insulin).Category: Insulin analogIndication: Treatment of diabetes (type I and 2).

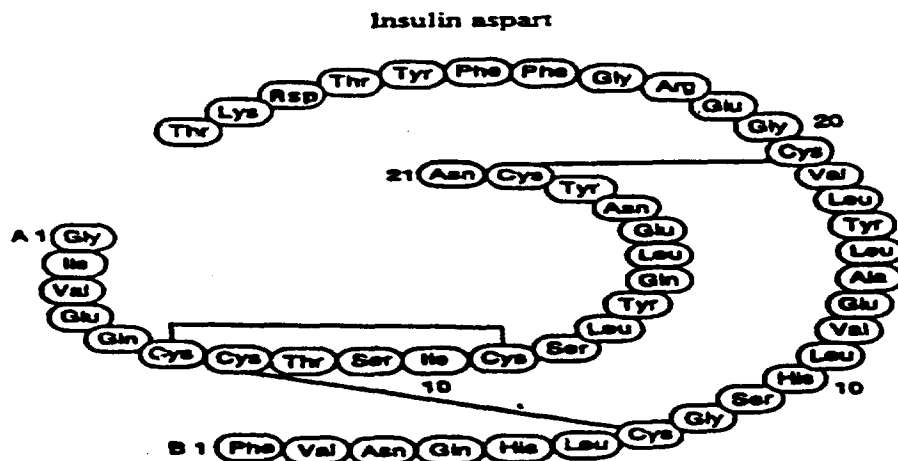
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Indra Antonipillai, Ph.D.

cc: NDA Arch
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HFD-510/steigerwalt/antonipillai/koller/jrhee
X14 insulin analog

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7/21/99*See Tefim Leach
memo to file*APPEARS THIS WAY
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REVIEW AND EVALUATION OF PHARMACOLOGY/TOXICOLOGY DATA**KEY WORDS:** Insulin, diabetes, glucose**Reviewer Name:** Indra Antonipillai**Division Name:** Division of Metabolic and Endocrine Drug products.**HFD#** 510**Review Completion Date:** July 21, 1999**IND/NDA number:** NDA 20-986**Serial number/date/type of submission:** September 16, 1998, original application**Information to Sponsor:** Yes (x) No () - (labeling)**Sponsor or agent:** Novo Nordisk Pharmaceuticals Inc., 100 Overlook Center, Princeton, NJ.**Manufacturer (if different) for drug substance:** Novo Nordisk A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark.**Drug:****Code Name:** Insulin Aspart (Insulin X-14, _____)**Generic Name:** N/A**Trade Name:** N/A**Chemical Name:** L-Aspartic acid-insulin human (Recombinant human insulin, DNA origin, β^{28} Asp-Insulin))**CAS Registry Number (if provided by sponsor):** N/A**Molecular Formula/ Molecular Weight:** $C_{256}H_{381}N_{65}O_{79}S_6/5825.8$ **Structure:****Relevant INDs/NDAs/DMFs:**

IND — (insulin X14, Novo Nordisk Pharmaceutical Inc)

IND _____

IND _____

IND: []
IND []Drug Class: Insulin AnalogueIndication: Treatment of diabetes (type I and 2)Clinical formulation (and components): 100 Units/ml of the active drug (insulin aspart) contains the following, at pH of 7.2-7.6:

Glycerin	16 mg/ml
Phenol	1.5 mg/ml
Metacresol	1.72 mg/ml
zinc	19.6 µg/ml
Disodium hydrogen phosphate dihydrate	1.25 mg/ml
Sodium chloride	0.58 mg/ml.

It is supplied as 10 ml vial, PenFill 3 ml cartridges, and Prefilled 3 ml syringes. Novolet is the approved name for prefilled syringe in Europe. The sponsor plans to use only the PenFill 3 ml cartridge, and Prefilled 3 ml syringe for this NDA.

Route of administration: subcutaneously in the abdominal wall, the thigh, or the upper arm.

Proposed clinical protocol or use: Insulin aspart is indicated for the treatment of diabetes mellitus as a rapid acting insulin analog. It should be taken immediately before meal, It should be used in regimens together with an intermediate or long-acting insulin. The physician, depending on the needs of the patient determines dosage, and it should be regularly adjusted according to blood glucose measurements. The individual insulin aspart requirement is usually between 0.5-1 U/kg/day,

Introduction and drug history: This drug at present is not marketed in any country.

There have been 18 completed clinical trials on this drug (to evaluate safety, efficacy, Pk and PD), of these 3 were conducted in USA/Canada, 12 in Europe, and 3 in Japan.

Studies reviewed within this submission: Some primary pharmacology studies, secondary pharmacology studies, ADME and PK/TK studies (1 and 12 month TK in rats and dogs), 4-week sc toxicity studies + 4-week recovery in rats, 1-year sc toxicity studies in rats, 4-week sc toxicity studies + 4-week recovery in dog, 3-month sc toxicity studies in dogs, 1-year sc toxicity studies in dogs, segment I & II fertility/teratology studies in rats, segment II teratology studies in rabbits, segment III peri-postnatal study in rats, genotoxicity studies (Ames test, mouse lymphoma cell forward gene mutation test, cultured human peripheral blood lymphocyte chromosome aberration test, ex vivo

UDS test in rat liver hepatocytes, and in vivo micronucleus test in mice). Also special toxicity studies (mitogenicity in MCF cells) are included

Studies not reviewed within this submission: Several studies were reviewed under IND
— These are incorporated in the appendix.

PHARMACOLOGY

A. INTRODUCTION

Insulin Aspart (X14) is an analog of human insulin, in which the amino acid, proline, in position β 28, has been replaced by aspartic acid. This modification was designed to decrease the self-association of the molecule. That is, X14 should have a reduced tendency to self aggregation due to ———. This might be the underlying mechanism whereby X14 would be absorbed rapidly, resulting in a fast onset of action, without losing other properties of human insulin.

Insulin aspart is produced by recombinant DNA technology in the yeast *sacharomyces cerevisiae* by Novo Nordisk A/S.

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The earlier drug manufacturing process was designated as process A (used in phase I/II studies). The later drug manufacturing process was designated as the new drug or process C (or the current drug).
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B. PHARMACOLOGIC AND PHARMACODYNAMIC STUDIES

I. The Potency studies of X14 vs human insulin:

Bioactivity Studies

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1. In ob/ob mice both X14 and human insulin (0.2 U/ml) decreased moderate hyperglycemia to a similar extent (decreased by 2.4 and 3.2 mmol/l resp). The molar potency of X14 is defined as 1U=6 nmol.
2. In the mouse blood glucose assay, the potency of 3 different batches of the X14 was similar (104-105% of human insulin) to international human insulin standard. Similar observations were noted in the rabbit blood glucose assay (potency was 94-104%).
3. In pigs equimolar doses of insulin aspart and human insulin (by iv administration) had similar effect on blood glucose, but aspart had a faster action after subcutaneous administration than human insulin.
4. The biological potency of X14 in the free fat cell was assayed relative to an internal standard. In this assay the effect of lipogenesis of an unknown dilution of X14 is compared to that produced by internal standard of human insulin. The potency of X14 was estimated to be 102.7% of human insulin standard.

These bioassay studies suggest that potency of X14 is similar to human insulin.

II. Receptor binding studies

1. Affinity of X14 for the Insulin Receptor and the IGF1-Receptor (study Number: 940272). This study was conducted by Novo Nordisk A/S, Denmark under GLP, and QA (signed and dated report).

Methods: Human insulin receptor (HIR) and IGF1-receptor (HIGFIR) were prepared by purification from transfected BHK-cells according to Adersen et al (JBC 267:13681-6, 1992). In brief, the relative affinity of the insulin analogs for the IGF1-receptor was determined by a classical displacement assay, where ^{125}I -IGF1 is displaced from the IGF1-receptor by increasing concentrations of insulin or analog. The relative affinity of the insulin analogs for the insulin receptor was measured by the binding of ^{125}I -labeled analog to a dilution series of the insulin receptor. The X14 used for the binding studies was Batch # E-24391.

Results: The displacement of ^{125}I -HIR with human insulin, and the displacement of ^{125}I -IGF1 from HIGFIR with IGF1 were used to verify the integrity of the receptor preparations. The binding curves and the affinities calculated by curve-fitting the data to the expression, for a simple one-site receptor binding are summarized below. The displacement of IGF1 from the IGF1-receptor was handled identically. The affinities of X14, and the X14 degradation products (or derivatives) are shown below (Tables 1 and 2).

**APPEARS THIS WAY
ON ORIGINAL**

Table 1

Analog	% Affinity
Human insulin	=100
X14	92±6
X14-β28- — Asp	83±6
_____	_____
_____	_____
_____	_____

Table 2.

Analog	Affinity relative to HI	Affinity relative to IGF1
Human Insulin	=100%	0.03%
IGF1		=100%
X14	132%	0.05%
_____	_____	_____
X14-β28 — Asp	86%	0.03%
_____	_____	_____
_____	_____	_____
_____	_____	_____

In summary, in the solubilised cloned receptors, the affinity of X14 for the insulin receptor is slightly lower (92%) than that of human insulin, whereas its affinity for the IGF1-receptor in this system is at least 1000 fold lower than that of IGF1. In contrast, the affinity of — for the insulin receptor is higher than that of human insulin.

Similarly in intact hepatoma human cells (in HepG2 cells in another study, reviewed under IND — see appendix), which compared the ability of X14 and human insulin to displace ¹²⁵I-labeled human insulin, or ¹²⁵I-labeled IGF-I (using a competitive binding assay), the affinity of X14 for the insulin receptor was 92.2% of human insulin (same as noted with solubilised receptors). In this system the affinity of X14 for the IGF-I receptor was 68.8% (range 62-76%) of human insulin, sponsor states that it is in the same range as human insulin.

III. Pharmacodynamic mixability study of X14 and human insulin:

A study in female pigs (n=4/group) was conducted with X14 alone, and with neutral protaphane (regular human insulin) alone, given as 2 separate injections sc (total dose of 0.15 U/kg), or 30:70 mixture of X14 and protaphane, given as a single injection sc (total dose of 0.15 U/kg, 5 min after mixing). At 180 min after dosing, the mean plasma glucose conc were slightly but significantly lower for a single injection vs given as two injections (p < 0.05). However, overall no significant differences in the plasma glucose (or maximal hypoglycemia) levels were observed with 2 methods (lowest plasma glucose levels were observed at 75 and 90 min with two methods resp), suggesting no pharmacodynamic interactions between the two insulin preparations.

SAFETY PHARMACOLOGY

A. General safety pharmacology studies

Several safety pharmacology studies (effects on GI, renal, cardiovascular, neurological, respiratory systems etc) were carried out in rats and mice at doses up to 100 U/kg (human therapeutic dose is 1 U/kg), and in cats and pigs up to 4 U/kg (due to higher sensitivity of the drug in these species). In all these studies the effects of human insulin (HI or HM(ge)) at high doses were also compared (see appendix). No significant differences were found in most studies, except in the following:

1. In anesthetized pigs (study # NN960235), a blood pressure reduction was observed at 4 U/kg of X14 (-15.53 vs 5.7 with human insulin controls at a similar dose, $p < 0.05$), however simultaneous infusion of glucose prevented this effect (2.7 vs 10.1 in vehicle controls), suggesting that the effect is due to hypoglycemia). Mean heart rate also decreased at 1 U/kg of X14 (-5.07 vs 18.8 with human insulin controls at a similar dose $p < 0.05$, but was not different at 4 U/kg -3.1 vs 1.1).
2. In rats, Increased diuresis was noted (at 4, 5 and 24 hrs post dose i.e. urine output was — vs — ml in controls), together with increased urinary excretion of electrolytes (Na 0.8 vs 0.1 in controls, Cl 1.2 vs 0.3 in controls) at 100 U/kg of X14. At 10-100 U/kg of X14, protein (— vs 2.9 in controls) and creatinine — vs 1.5 in controls) were increased at 5 hrs.
3. In mice, single iv administration of X14 prolonged the hexobarbital-induced sleeping time (at 10-100 U/kg — vs 51 min in controls, by 2-fold, which was also noted with 100 U/kg of HM(ge) i.e. 110 min, the positive control chlorpromazine increased it to 137 min, all $p < 0.01$). Also in another assay of ethanol induced sleeping time in mice, 100 U/kg dose of both X14 or HM(ge) increased the mortality in animals (100% vs 25% in controls), and all doses of the drug had tendency to increase the ethanol-induced sleeping time (— vs 98 min in controls), which suggests some interaction of X14 with ethanol.
4. The drug slightly reduced spontaneous activity (in 2 of 6 mice at 100 U/kg of X14, which was also noted in 2 of 6 mice with 100 U/kg HM(ge).

All these effects could be prevented by concomitant administration of glucose, suggesting that these may also be due to hypoglycemia, and these were generally also observed with human insulin (HM(ge)).

B. Neurological/Cardiovascular studies:

Effects of X14 on the Autonomic Nervous System in the Anaesthetized Cat (Study No. NN960236). This study was conducted by ' ————— ' under GLP standards.

Methods: A total of 9 male cats, 9-15 months of age, were anesthetized with pentobarbital and the stage of anesthesia was maintained by a continuous infusion of

pentobarbital for the duration of experiments. Three cats were administered either vehicle or X14 (Batch X149602) at doses of 0.4, 1.0 and 4.0 U/kg. Another three cats were used for the evaluation of regular human insulin (Batch H01617 at doses of 0.4, 1.0 and 4.0 U/kg), while the rest of 3 cats were infused glucose continuously at a rate of 14 mg/kg/min from the first administration of X14. In all experiments, epinephrine (1 μ g/kg) was injected intravenously, as a positive control. The femoral arterial blood pressure, heart rate, blood gas, and EKG were recorded.

Results: There were no marked or sustained changes in mean blood pressure (BP) or in heart rate (HR) following treatment with X14 or regular human insulin (HI) in the presence or absence of glucose infusion. There was no clear dose dependent difference between human insulin and X14 in respiratory rate. However, a statistically significant reduction ($P < 0.05$) in respiratory rates between the two insulins at 0.4 U/kg in the presence of glucose infusion was observed (with X14 -17.97 vs with HI (ge) - 8.37). The pharmacological significance of this difference is not clear since it was not drug dose dependent. To test potential overt changes in the parameters during bilateral carotid occlusion (BCO) and norepinephrine infusion (Nor), both regular human insulin (HI) and X14 were not remarkably different. The findings are summarized in Table 3 below.

Table 3.

Treatment and iv Drug Dose	Group mean maximum % change from immediately pre-dose			
	MP after BCO	HR after BCO	MP after Nor	HR after Nor
Vehicle	28.53	2.47	45.70	25.03
HI (0.4 U/kg)	28.73	4.30	57.70	28.00
HI (1.0 U/kg)	23.70	1.67	56.67	26.13
HI (4.0 U/kg)	20.50	1.20	60.83	24.20
X14 (0.4 U/kg)	20.77	3.67	59.10	12.63
X14 (1.0 U/kg)	25.57	4.33	67.23	12.00
X14 (4.0 U/kg)	19.90	2.27	74.63	14.40
Vehicle	23.70	0.80	46.80	11.83
X14 (0.4 U/kg)*	14.83	0.70	65.77	13.73
X14 (1.0 U/kg)*	18.53	1.43	67.70	11.23
X14 (4.0 U/kg)*	16.90	1.77	65.10	10.40

BCO = Bilateral carotid occlusion,

Nor = Norepinephrine

HI = Regular human insulin

X14 = Insulin aspart

* = indicates glucose infusion.

C. Motor activity studies

Effects of Insulin Aspart on Locomotor Activity in CD Rats (Study No. NN960224)

This study was conducted by _____ under
_____ Principles of GLP.

Methods: Male CD rats (8/group) were given either vehicle (1000 mg % zinc _____ 200 mg % disodium phosphate and 3200 mg % glycerol _____ or X14 (batch number X149602) at 1, 10, or 100 U/kg intravenously at a volume-dosage of 1.0 ml/kg body weight. Satellite groups of rats (8/group) were used for the evaluation of regular insulin or positive control agents such as chlordiazepoxide (10 mg/kg) or D-amphetamine sulfate (1mg/kg). Rats were observed for clinical signs of toxicity, body weight, food consumption, motor activity measurement (photobeams) for a single injection of each administration.

Results: The acute intravenous administration of X14 to rats at dosages of 1 to 100 U/kg did not produce significant differences in locomotor activity during the 15-minute period. No significant differences in motor activity were found between those animals which received X14, 100U/kg or regular insulin, although positive agents altered motor activity as expected. There were no remarkable difference between the control group and X14 treated groups in other parameters such as body weight and clinical symptoms.

Summary of pharmacology

The primary pharmacology suggest that potency of X14 is similar to human insulin. In ob/ob mice both X14 and human insulin (0.2 U/ml) decreased moderate hyperglycemia to a similar extent (by 2.4 and 3.2 mmol/l resp). In the mouse blood glucose assay, the potency of X14 was similar (104-105%) to international human insulin standard. The faster action of the drug was shown in pigs, where equimolar doses of insulin aspart and human insulin had similar effects on blood glucose (by iv administration), but aspart had a faster action after subcutaneous administration than human insulin (____ vs _____ hrs with human insulin, $p < 0.05$). Receptor binding studies indicated that the affinity of X14 for the insulin receptor is slightly lower (92%) than that of human insulin (100%), whereas the affinity of X14 and insulin for the IGF1-receptor in this system is 0.03% and 0.05% resp (about 2000 fold lower), than that of IGF-1 (100%).

Several secondary or safety pharmacology studies (up to 100 U/kg doses in rats and mice, and up to 4 U/kg in pigs) were conducted, no significant differences were observed except the drug caused a blood pressure reduction at 4 U/kg in anesthetized pigs (-15.53 vs 9.9 in controls). In rats, increased diuresis was noted together with increased urinary excretion of electrolytes at 100 U/kg of X14. At 10-100 U/kg of X14, protein _____ vs 2.9 in controls) and creatinine _____ vs 1.5 in controls) were

increased. In mice, single iv administration of X14 prolonged the hexobarbital-induced sleeping time at 10-100 U/kg by 2-fold, suggesting that the drug can possibly alter the hepatocyte enzymes. Both the drug and human insulin at 100 U/kg, slightly reduced spontaneous activity in 2 of 6 mice. All these effects could be prevented by concomitant administration of glucose, suggesting that these may also be due to hypoglycemia, and were generally also observed with human insulin (HM(ge)).

PHARMACOKINETICS/TOXICOKINETICS

The pharmacokinetics of X14 have been studied in SD rats and Beagle dogs after single dose (following iv and sc administration) and after multiple doses (sc administration) in SD rats, beagle dogs and cross breed of female land race pigs. In these studies comparisons were also made with human insulin. **All of the preclinical PK/TK studies were analyzed using the _____, which measures both insulin aspart and native human insulin.** Sponsor states that "only very recently, it was possible to produce an antibody which specifically binds to the rapidly acting B28(ASP)-human insulin analog, without any binding to native human insulin". However, it is not mentioned whether this antibody binds to rat, dog or pig insulin also, or is only specific for human insulin.

ADME- Absorption

Single dose studies:

Intravenous studies in both rats (study # 960550) and dogs (study # 960548): Only minor differences were observed here. In both rats (1 U/kg) and dogs, following iv administration of X14 (¹²⁵I Tyr A14), both X14 or human insulin had plasma elimination half lives of 12 and 14 min in rats, and 11 and 13 min in dogs resp. The fast elimination was due to its fast clearance which was 44 ml/min/kg in rats (human insulin was 58 ml/min/kg). The mean residence times were below 30 min. Cmax for both X14 and human insulin were comparable (17.4 and 17.7 nmol resp)

Subcutaneous studies in both rats (study # 960550) and dogs (study # 960548):

Following sc administration (1 U/kg), the plasma elimination half lives were 22 and 67 min in rats and dogs resp (human insulin was 23 and 57 min resp). The time to reach the maximum conc were 15-30 and 30-90 min in rats and dogs resp. The increase in half life following sc (vs iv) may be due to prolonged absorption from the injection site.

The bioavailability of X14 and human insulin in rats were 82% and 90% resp. The sc bioavailability of X14 in male and female rats were 108% and 90% resp. In dogs this was 100% for both X14 or human insulin.

Neither rat or dog indicated the faster onset of action of X14 or faster sc absorption, this could be shown in pigs, and later in man. The explanation may be that structural differences in the subcutis (which consist of less lipid) exist in dogs and rats, than in

pigs and human. These differences result in generally faster kinetics after sc absorption.

Table 4. Comparative PK differences between species (for both X14 and human insulin (HM)(ge) are shown here.

Species (dose)	Rat (6 U/kg)		Dog (1 U/kg)		Pig (0.125U/kg)		Man	
	X14	HM	X14	HM	X14	HM	X14	HM
$T_{1/2}$ (min)	22	23	67	57	77	121	76	122
C_{max} (nM)	1.7	1.8	3.2	2.9	0.20	0.12	0.25	0.10
T_{max} (min)	15	15	46	60	73	99	52	145
Cl (ml/min/kg)	44	58	55	48	51	52	20	20

Pharmacokinetics of intravenous and subcutaneous X14 and human insulin in female crossbred LYYD pigs (Study # 950219, and Study # 950475) :

Methods: Pigs have close similarities to humans with respect to composition of subcutaneous tissue, therefore this species was chosen for the current studies. Five pigs (mean weight 81 kg) received X14 (insulin aspart batch # 06694), or human insulin (batch # 05994, mean weight of animals 86.2kg) of 0.025 U/kg in an ear by iv (via a catheter) or sc 10 U (or 0.125 U/kg) on each side of neck (125 I-Tyr A14 labeled insulin, batch # Q95013), and plasma samples were obtained at various time points.

Results: PK with both X14 and human insulin after iv were similar in pigs (C_{max} 360 and 456 pM resp, CL 0.019 and 0.021 l/kg resp, V 0.126 and 0.096 l/kg resp). Effects on plasma glucose were also not significantly different. However, after sc administration the disappearance rates were significantly different at all time points during 6 hrs observed. The X14 disappeared at a significantly higher rate from the subcutis than human insulin (— vs — hrs with human insulin, $p < 0.05$ at all time points, see appendix).

Multiple dose studies: In both rats (study # 960551), and dogs, drug was administered daily for 8 days (twice daily, 4 hrs apart) and samples were collected on day 8 and compared with those of single dose study in rats. In dogs, samples were obtained on day 1 and 8. There was a dose linearity in PK and no accumulation was observed on day 8 in both species, and generally no differences vs human insulin in PK were observed.

Toxicokinetics: One year TK studies (a twice daily dose) were conducted using the old process drug (process A), but 1-month studies (a twice daily dose) were carried out with new process drug (process C). As indicated earlier, that a change in manufacturing process was implemented in the drug development —

The earlier drug manufacturing process was designated as process A, the later drug manufacturing process (or the current drug to be used clinically) is designated as process C. One year TK studies which were designed to dosing twice daily were changed to dosing once daily in both rats and dogs (after week 26 in rats, and at week 29 in dogs), due to hypoglycemia. All of the preclinical PK/TK studies were assessed using the _____ (which measures both insulin aspart and native human insulin). Thus due to lack of specific assays for rat/dog C-peptides, the endogenous insulin levels for various species could not be calculated, and therefore AUC values could not be determined.

Rat

1-Month TK studies in rats (Study # 960450): Four groups (n=10/sex/group) were given the new process drug (X14) twice daily by subcutaneous injections at doses of 0, 5, 25, 100 U/kg/bid for 4 weeks (or total dose of 10, 50, 200 U/kg/day). The fifth group received the old process drug (X14) at twice daily dose of 100 U/kg/bid (or total dose of 200 U/kg/day). Blood samples were collected on days 1 and 28, at 0, 1, 3, 4, 5, and 8 hr. Method details are provided in the 28-day toxicity study in rats. Highest insulin X14 levels were noted at 1 and 5 hrs. These are shown in Table 5. No significant differences were noted with new vs old X14.

Table 5. Mean plasma levels of new and old process X14 drug in 28-day subcutaneous toxicity study in rats (n=9/sex/dose), at doses of 10, 50, and 200 U/kg/day.

Dose U/kg/day	Day 1 (nM)		Day 28 (nM)	
	Mean plasma conc at 1 hr	Mean plasma conc at 5 hr	Mean plasma conc at 1 hr	Mean plasma conc at 5 hr
Males				
New X14-10	1.9	8.7	6.8	9.2
New X14-50	44.1	81.9	62.2	78.2
New X14-200	216.4	334.0	261	440
Old X14-200	226.3	296.9	229	314
Females				
New X14-10	2.8	0.7	4.4	3.9
New X14-50	38.4	31.5	49.2	74.0
New X14-200	202.7	189.6	199	269
Old X14-200	141.7	142.4	238	334

One year TK study in rats (Study # 960270): Four groups (n=12/sex/group) were given the old process drug (X14) twice daily by subcutaneous injections at doses of 0, 5, 25, 100 U/kg/bid for 52 weeks (or total dose of 10, 50, 200 U/kg/day). The fifth group received the HM(ge) at twice daily dose of 100 U/kg/bid (or total dose of 200 U/kg/day). Blood samples were collected on days 1 and after weeks 13, 26, and 52, at 0, 1, 3, 4, 5, and 8 hr. Method details are provided in the 1-year toxicity study in rats (see page 23).

Highest insulin X14 levels were noted at 1 hr (M+F _____ nM at 10, 50, and 200 U/kg/day resp) and 5 hrs. No gender differences were noted. Linearity in Cmax and doses were observed, however no tabular data were provided, only figures were given (see appendix).

Dog

1- month TK study in dogs (Study # 960452). Four groups (n = 4/sex/group) were given the new process drug (X14) twice daily by subcutaneous injections at doses of 0, 0.25, 0.5 and 1.0 U/kg/bid for 4 weeks (or total dose of 0.5, 1.0 and 2.0 U/kg/day). Blood samples were collected on days 1 and 28, at 0, 1, and 4 hrs after first dose, and 1 and 4 hrs after second dose. Method details are provided in the 28-day toxicity study in dogs (see page 29). Highest insulin X14 levels (pM) were again generally noted at 1 and 5 hrs. These at 1 hr were as follows: Dose linearity was noted in both sexes in dogs.

	Males	Females (at 0, 0.25, 0.5, and 1.0 U/kg/bid)
Day 0	180, 195, 337, 740,	175, 185, 323, 709
Day 28	123, 168, 292, 940	79, 200, 344, 940

1-year TK study in dogs (Study # 960130, test facility Novo Nordisk Park, Malov): Four groups (n=4/sex/group) were given the old process drug (X14) twice daily by subcutaneous injections at doses of 0, 0.25, 0.5 and 1.0 U/kg/bid for 52 weeks (or total dose of 0.5, 1.0 and 2.0 U/kg/day). The fifth group received the recombinant human insulin HM(ge) at twice daily dose of 1U/kg/bid (or total dose of 2 U/kg/day for comparative purposes) for 52 weeks. Blood samples were collected on days 1, 28, weeks 13, 26, at 0, 1, 4, 5, and 8 hr, and in week 52 at 0, 1, 4, 6, and 8 hrs. Method details are provided in the 1-year toxicity study in dogs (see page 33). Highest insulin X14 levels were generally noted at 1 hr (M+F _____ nM at 0.5, 1.0, and 2.0 U/kg/day resp). Mean tabular data for groups were not presented, except in figures. Dose linearity was noted in both sexes on day 0, and in weeks 13, 26, 29 and 52 (see figures in appendix), and no sex differences were observed in TK.

Toxicokinetics from segment II study in rabbits (Study # 960419, test facility Novo Nordisk Park, Malov): Four groups of 4 mated satellite female rabbits were given the old process drug (at 0, 0.5, 1.5 and 3 U/kg/bid) or recombinant human insulin (HM(ge) at 0.5, 1.5 and 3 U/kg/bid for comparative purposes), subcutaneously twice daily (4-hrs apart), from days 6 to day 18 of gestation. Surviving females were sacrificed on day 29 PC and necropsied. Blood samples were collected on days 6 PC (the first day of treatment) at 0, 1, 4, 5, and 8 hr. Method details are provided in the segment II toxicity study in rabbits (see page 45).

Highest insulin X14 levels were generally noted at 1 hr and 5 hrs. Dose linearity was observed.

The mean values for groups with X14 or HM(ge) at 0.5, 1.5 and 3 U/kg/bid were (nM):

Groups	X14	HM(ge)
1-hr	1.39, 4.56, 12.98	0.85, 6.03, 23.63
5-hr	1.49, 4.93, 18.83	1.17, 4.38, 17.19

Distribution/Metabolism/Excretion of X14

Distribution of X14

The distribution of radioactivity in male Sprague Dawley rats and in pregnant female rats, after a single sc dose of ^{125}I (Tyr A14)-X14. Study # 950338. Test facility

Methods: Distribution of ^{125}I (Tyr A14)-X14 (1 U/kg) was examined after a single sc dose of the drug (batch # 06694) to male SD rats (3 rats/group), and to healthy non pregnant and pregnant rats (at 15 day of gestation), in 3 rats /group. Rats were sacrificed at 0.5, 2, 4, 24 and 168 hrs after drug administration and radioactivity in plasma, various tissues, and urine/feces/cage rinses was determined by _____

The distribution of radioactivity was also determined by _____

_____ Milk secretion of radioactivity was also measured.

Results: The radioactivity was widely distributed into most tissues and organs. Highest radioactivity in most organs and tissues was observed at the earliest time point of 0.5 and 2 hrs after dosing, and decreased with time, and by 168 hrs very little was observed. The mean concs in the plasma were higher (M 7.6, F 11.4 pmol eq/ml at 2 hrs) than in several organs and tissues. Highest concs were observed in thyroid (at 2 hrs in males 2440, at 24 hrs 20700 pmol equivalent/g tissue), at injection site (at 2 hrs in males 15.6, females 14.9 pmol/g tissue), in aorta (M 12.0, F 13.6), vena cava (M 11.8, F 15.9), GI tract (M 22.7, F 19.9), kidneys (M 10.1, F 11.1), pituitary (M 2.9, F 20.6), and trachea (M 4.4, F 4.3), medium concs were observed in prostate/ovaries (7.3 & 6.5) and skin (5.7 & 6.0). Lowest concs were observed in brain and white fat, see appendix. Thyroid (321 fold), aorta (1.6 fold), bladder (1.2 fold), GI contents (3 fold), kidneys (1.3 fold) and vena cava (1.6 fold) had higher conc than circulation (plasma). The drug accumulates in thyroids. Similar pattern of distribution were found in maternal tissue, i.e. the peak levels were attained at 0.5 and 2 hrs (except for kidneys which had 52.2 pmol/g), and highest concs were observed in plasma (at 0.5 hrs, 10.1 in plasma vs _____ in tissues, fetuses 1.0 pmol/g). The radioactivity in fetuses was 6-10% of plasma. The radioactivity decreased by 24 hrs, and in fetuses at 24 hrs it was 0.05 pmol/g. Mean radioactivity in milk was 2.9, 42.3, 73 and 1.8 pmol/ml at 0.5, 2, 4, and 24 hrs resp. The ratio of AUC (0-24 hrs) of milk to plasma was 30:1. Most of the radioactivity in milk was _____ precipitable (69-84%). These studies suggest that the placental transfer of the drug (X14) is small but the drug is incorporated into the milk of lactating animals. The drug does not accumulate except in thyroids which is due to release of ^{125}I during degradation of the labeled molecule.

Protein binding effects of X14 (using an _____ method) were examined in EDTA treated pooled plasma from rats, dogs, rabbits, pigs and humans, using 10 pM to 10-100 nM concs of the drug (batch # C96023) with ^{125}I -(Tyr A14) X-14. In rats, the mean binding was 22-29% at low doses of 10 pM (at higher doses of 1-100 nM, the binding was reduced to 0-17% in rats), with no sex differences. In dogs (males or females), the mean binding was negligible (0-2%). In female pigs the mean binding was 23% at low doses of 10 pM (at 1-100 nM, it was 11-15% in pigs). In two female rabbit studies (samples obtained at different times of the year), the binding was 70-83% and 18-26% resp, sponsor states that this may be due to proteins or due to antibody formation in rabbits. In human plasma (from 4 healthy volunteers, 2 males + 2 females, by equilibrium dialysis), the mean ratio of free conc to total plasma conc of the drug was 0.9-1.01 at 10 pM to 10 nM, suggesting protein binding of 0-10%. Thus highest protein binding of 10% was observed in human plasma, with no sex differences. To summarize, protein binding of X14 was low in all species tested (rat, dog, pig and man at 10 pM to 10 nM). However, the comparative protein binding of regular human insulin in human plasma vs X14 was not provided.

The distribution of the drug (6 pM to 6 nM in animals, and 600 pM to 6 nM in humans) between erythrocytes and plasma was examined. In human blood the fraction of the drug in human whole blood that was bound to the red blood cells ranged from _____ (the mean counts in blood/counts in plasma ranged from 0.65 to 0.68), with no sex differences in distribution in the whole blood were noted, and it largely remained in extracellular fraction of the whole blood. Similarly, in animals (dogs, rats, pigs and rabbits), very small amounts of the drug were found adhering to red blood cells and it also largely remained in the extra cellular fraction of the whole blood.

Metabolism of X14

Following subcutaneous and iv administration of ^{125}I (Tyr A14)-X14 to rats (study #950337) there was rapid and complete absorption of the radioactivity in the systemic circulation (the bioavailability in male and female rats was 101 and 106% resp). The proportion of the radioactivity precipitable by _____ (the intact drug) was determined in this study. At 1-4 hrs, the precipitable portion of radioactivity declined to 13-17% in rats, after that it increased to 83-87% by 48 hrs, this suggests extensive and rapid metabolism of the radioactive ^{125}I -X14 to smaller peptides, and also possibly to ^{125}I iodide, which was incorporated into host proteins. The rapid metabolism was confirmed in plasma, urine, feces and tissue samples. There were no sex differences. These catabolism products were not identified but assumed to be amino acids and peptides resulting from the drug or the host proteins, following subsequent incorporation of the label (by endogenous metabolism). Only very polar metabolites were detected in plasma and selected tissues, these are mostly smaller peptides, and also possibly to ^{125}I iodide and ^{125}I -tyrosine. Thus the drug is rapidly degraded following sc absorption.

The enzymes responsible for degradation of X14 are unknown. It is suggested that it is degraded in a manner similar to human insulin (by insulin protease and or insulin degrading enzyme, and possibly by protein disulfide isomerase), leading to formation of human insulin metabolites which are not active. No in vitro drug-drug interaction studies have been conducted with X14, since these have not been reported with human insulin.

Excretion of X14

The mean recovery of radioactivity after 168 hrs was 102% and 104% in male and female rats resp (Study # 950338). In both sexes the most of the radioactivity was excreted in the urine, and higher urinary excretion was observed in females (87% of the dose was excreted vs 77 % in males), while 10.4% and 8.7% was excreted in feces (in males and females resp), and the rest in organs and carcass, 17.3 and 6.7 % resp

Comments and Conclusions on ADME: Both iv and sc, as well as summary on toxicokinetic studies (sc, from 1- and 12-month toxicity studies) in rats and dogs are presented here.

Following iv administration of X14 to both rats and dogs, X14 had plasma elimination half lives of less than 15 min (for human insulin it was 14 min). The fast elimination was due to its fast clearance which was 44 ml/min/kg (human insulin was 58 ml/min/kg). The mean residence times were below 30 min. Cmax for both X14 and human insulin were comparable (17.4 and 17.7 nMol resp). Following sc administration, the plasma elimination half lives were 22 and 67 min in rats and dogs resp (for human insulin these values in rats and dogs were 23 and 57 min resp). The time to reach the maximum conc were 15-30 min in rats, and 30-90 min in dogs. The increase in half life following sc (vs iv) may be due to prolonged absorption from the injection site. In humans the half life of X14 was 76 min vs 122 min for the regular human insulin. The bioavailability of X14 and human insulin after sc dosing in rats were 82% and 90% resp. In dogs this was 100% for both X14 or human insulin. Neither rat or dog indicated the faster onset of action of X14 or faster sc absorption, because the structural differences in the subcutis (which consist of less lipid) exist in dogs and rats, than in pigs and human (which result in generally faster kinetics after sc absorption). No accumulation of the drug (or for human insulin) was observed in rats or dogs after administration for 8 days. The TK studies (4 or 52 weeks) showed maximal conc at 1 hr after SC injection, and general linearity in the plasma conc after 1 hr were observed for both rats and dogs, with no sex differences. In rats the plasma concs (1-hr values) at 100 U/kg/bid (the highest dose) in 4-week toxicity studies were 261 and 199 nM for male and females resp. In 52-week toxicity studies these were 265 and 334 nM for male and females resp at 100 U/kg/bid, the dose was switched to 75 U/kg/once a day in this study due to hypoglycemic death in animals). In dogs the plasma concs (1-hr values) at 1 U/kg/bid in 4- and 52-week toxicity studies were 0.94 nM (in both sexes) and 3.2-3.9 nM (in

males and females resp) resp. Again in 52-week dog studies, the dose was switched to once a day, due to hypoglycemia.

X14 was widely distributed throughout the body, the peak tissue levels were attained at 0.5 and 2 hrs after sc dosing in male rats, and decreased with time. By 168 hrs very little was observed. Highest concs were observed in thyroid (321 fold of plasma conc), aorta (1.6 fold), bladder (1.2 fold), GI contents (3 fold), kidneys (1.3 fold) and vena cava (1.6 fold of plasma conc). The drug appears to accumulate in thyroids, which is most likely due to release of ^{125}I from the parent compound. Medium concs were observed in prostate/ovaries and skin. Lowest concs were observed in brain and white fat. Similar pattern of distribution was noted in healthy pregnant rats at 15 days of gestation, and highest concs were observed in plasma (at 0.5 hrs, 10.1 in plasma vs — in tissues, fetuses 1.0). The radioactivity in fetuses was 6-10% of plasma, which decreased by 24 hrs. The placental transfer of the drug (X14) was small, but the drug is incorporated into the milk of lactating animals (the radioactivity in milk was 2.9, 42.3, 73 and 1.8 pmol/ml at 0.5, 2, 4, and 24 hrs resp). The ratio of AUC (0-24 hrs) of milk to plasma was 30:1.

In rats, the mean protein binding was higher at low doses of 10 pM (22-29% vs at higher doses of 1-100 nM of 0-17%, with no sex differences). In dogs it was negligible (0-2% M or F). In female pigs the mean binding was 23% at low doses of 10 pM (at 1-100 nM, 11-15% in pigs). In two female rabbit studies (samples obtained at different times of the year), the binding was 70-83% and 18-26% resp, this may be due to proteins or due to antibody formation in rabbits. In human plasma (from 4 healthy volunteers, 2 males + 2 females, by ————, the mean ratio of free conc to total plasma conc of the drug (10 pM-10nM) was 0.9-1.01 at 10 pM to 10 nM, suggesting protein binding of 0-10%. Overall low protein binding of X14 was observed (in pooled plasma) in rat (0-29%), dog (0-2%), pig (11-23%), and man (0-10%). Thus, highest protein binding of 10% was observed in human plasma, with no sex differences.

The drug is rapidly and extensively metabolized to very polar metabolites (detected in plasma and selected tissues, these are mostly smaller peptides and also possibly to ^{125}I iodide and ^{125}I -tyrosine). The rapid metabolism was confirmed in plasma, urine, feces and tissue samples. There were no sex differences. These catabolism products were not identified but assumed to be amino acids and peptides resulting from the drug or the host proteins. Most of the drug is excreted in the urine, and higher urinary excretion was observed in females (77 and 87% of the dose resp in males and females), while 10.4% and 8.7% was excreted in feces, and the rest in organs and carcass, 17.3 and 6.7 % resp.

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TOXICOLOGY (Single and repeat dose toxicology)

Acute Toxicity

RatsEffects of X14 After Single Subcutaneous Injection in SD Rats (Study No. 950382)

This study was conducted by Novo Nordisk, Denmark, in accordance with the OECD guidelines.

Methods: Six groups of SD rats (5/sex/group) were administered a single dose of either vehicle (0.15% phenol, 0.172% M-cresol and 1.60 % glycerol _____) or X14 (new drug, Batch No. C96014) subcutaneously at doses of 62.5, 250, 1000, 4000 U/kg, or X14 (old drug and batch No. 07194).

Results:

Mortality and Clinical Signs: There were no deaths in any of the groups throughout the observation period. All animals including controls showed a decreased motor activity at 30 min. In the high dose groups slight ptosis and piloerection were noted in 30 min after dosing. At 2 hrs, only the animals in the high dose group (all animals) had a decreased motor activity (vs none in controls)

Body Weight and body Weight gain: There were no inter-group differences in body weight gain for 2 week-period.

Conclusion: A NOAEL could not be established in this study. The highest non-lethal dose level was greater than 4000 U/kg in both sexes of SD rats. No differences in acute toxicity studies were observed between the old and new drug.

DogsEffects of X14 After Single Subcutaneous Injection in Beagle Dogs (Study No. 950377)

This study was conducted by _____, in accordance with the OECD guidelines.

Methods: Four groups of one male and one female beagle dog were given new drug X14 (Batch No. C96015) subcutaneously at doses of 4, 8, 16, 32, 64 U/kg, or old drug X14 (Batch No. 06994) sc at 64 U/kg. Some dogs received more than one dose 21 days after the initial dose so that washout period was at least 3 weeks.

Results:

Mortality and Clinical Signs: There were no unscheduled deaths. The high dose produced uncoordinated movement and an unsteady gait at 15 min after dosing, of which plasma glucose was 1.67 mmol/l. There were clinical signs, indicative of hypoglycemia at other dosage levels.

Body Weight and Food Consumption: There were no treatment effects on the parameters.

Hematology and Biochemistry: There was clearly a time and drug-dose dependent hypoglycemia. All other individual values were considered characteristic of this species, which appeared to be unaffected by the treatment.

Macroscopic pathology and organ Weights: There were no findings considered to be related to treatment with X14 or regular human insulin at post mortem examination. All absolute and relative organ weights were in normal range of the species.

Conclusion: The highest doses (up to 64 U/kg) of X14 were well tolerated in dogs. Decreases in blood glucose were observed in all animals after the treatment. No differences in acute toxicity studies were observed between the old and new drug

In summary, in acute toxicity studies, the highest single dose of X14 tested in rats (4000 U/kg of old and new drug), and dogs (64 U/kg of old and new drug) was not lethal in any species, as no mortalities were noted. All doses produced hypoglycemia in animals. At 4000 U/kg in rats, slight ptosis, piloerection, and decreased motor activity (vs none in controls) was noted. In dogs, the high doses (64 U/kg) produced uncoordinated movement and an unsteady gait at 15 min after dosing (indicative of hypoglycemia).

REPEAT DOSE TOXICITY STUDIES

Four Week-Toxicity Studies in Rats after Twice Daily Subcutaneous Injection, followed by a 4-week recovery period (Study No. 950378):

Sponsor's ID Study # 950378

Amendment #, Vol #, and page # : volume 17, page 114

Conducting laboratory : _____

Date of study initiation: June 26, 1996

GLP compliance: Yes

QA Report: Yes (X) No (), Is the evaluation based on a final, QA report: Yes

Methods: A change in manufacturing process was implemented in the drug development _____

— The later drug manufacturing process was designated as the new drug or process C (or the current drug). This study compared the effects of the new drug (at 0, 10, 50, and 200 U/kg/day) vs the old drug (at 200 U/kg/day) in rats for 4 weeks. At the end of treatment period all groups were sacrificed, except the control and the high dose groups (with both old and new drugs) which were kept for additional 4 week of drug free recovery period.

Dosing information:**species:** CrI:CD BR rats**#/sex/group or time point:** 10/sex/group**age:** ~28 days old**weight:** Males 72-84 g, females 65-75 g.**satellite groups used for toxicokinetics or recovery:** 9/sex/group were used for TK and 5/sex/group were used for reversibility studies**Dosage groups in administered units:** Four groups (Ten rats/sex/group) were given either vehicle (0.15% phenol, 0.172% M-cresol and 1.60 % glycerol _____) or X14 (new process drug) twice daily by subcutaneous injections at doses of 5, 25, 100 U/kg/bid for 4 weeks (or total dose of 10, 50, 200 U/kg/day). The fifth group received the old process drug (X14) at twice daily dose of 100 U/kg/bid (or total dose of 200 U/kg/day)**Route, form, volume, and infusion rate:** Subcutaneous injections were given twice daily, 4 hrs apart, at a volume of 0.5 ml/kg, for 4 weeks.**Drug, lot #:** X14 new process drug, Batch # C96016, C96015 and C96014. X14 old process drug, Batch # 07094**Formulation/vehicle:** Vehicle was 0.15% phenol, 0.172% M-cresol and 1.60 % glycerol.**Times at which Observations are made:****Clinical signs:** Daily**Body weights:** At The time of allocation to groups, on the day of Tx and once a week thereafter.**Food consumption:** Weekly.**Ophthalmoscopy:** Before treatment and during week 4 of treatment.**Hematology:** Prior to the first dose and at the start of week 4**Clinical chemistry:** Prior to the first dose and at the start of week 4**Urinalysis:** Overnight urine samples were collected during week 4.**Gross pathology:** At sacrifice.**Organs weighed:** Organs weighed are listed in histopathology Table in appendix.**Histopathology:*** At sacrifice.**Toxicokinetics:** Days 1 and 28, at 0, 1, 3, 4, 5, and 8 hr.**Other (antibody determination):** Day 1 and at post mortem.

*note: List of tissues examined by histopathology are given in tabular form, in the appendix

Results:**Mortality and Clinical Signs:** One male in the high dose group was found collapsed after the second dose of the day, on day 7 of treatment. The death appeared to be treatment-related. In addition, there was a death of one female in the 50 U/kg/day group, which was sacrificed in Week 4 due to an accidental eye injury.

Body Weight and Food Consumption: There was a non dose related increase (3 -13%) in body weights, in all treated groups, except in females in the 50 U/kg/day group (in which there was approximately 5% reduction). Body weights were significantly increased in males at a high dose, with a greater effect with old X14 (13% vs 7% with new X14). During the 4-week recovery period the body weights were lower at 200 U/kg/day (males by 2% and 10%, females by 32% & 20% with new and old X14 resp). Food consumption was increased in all treated males (by 6-13%), with no dose response relationship, but not significantly altered in females, and during the 4-week recovery period it was similar to controls. Food utilization was similar in treated and controls, but during recovery period food utilization was inferior in animals (both sexes) due to lower body weights.

Water consumption: This was increased in all treated males (by 9-20%) and slightly in females (by 1-6%) , and also remained increased in recovery periods (males by 5-15%, females by 3-8%).

Hematology: Packed cell volume, hemoglobin, RBC counts and mean corpuscular hemoglobin concentration(MCHC) were not different from the control before and after the drug treatment. Mean corpuscular hemoglobin and mean corpuscular volume were statistically elevated after the treatment, but the increase was under 5%.

Biochemical Parameters: There were no remarkable findings in the parameters except plasma glucose concentration which was reduced in time and drug-dose dependent manner, as shown below. Recovery in glucose levels occurred quite rapidly in the lower drug groups, but more slowly at higher doses. Plasma glucose conc with the old X14 (at 200 U/kg/day) were generally similar (males 9.8, 4.6, 4.0, 4.4, 3.8 and 3.8, females 10.3, 4.9, 3.9, 4.5, 3.9, and 3.4 at 0, 1, 3, 4, 5 and 8 hrs resp) to the new X14 (see Table 6 below).

Table 6.

Time After X14 Treatment(hr.)	Subcutaneous X14 Daily Dose (U/kg/day) in Male/Female Ctrl: Rats			
	Control	10	50	200
0(Predrug)	8.23/9.01*	9.22/8.97	9.60/8.92	9.90/10.40
1	8.16/9.07	4.38/5.61	4.96/5.51	4.57/5.27
3	8.29/7.72	9.40/7.92	4.01/4.85	3.66/4.03
4	8.77/9.21	9.73/9.06	8.03/7.94	4.50/6.25
5	8.27/9.07	4.68/4.68	4.79/4.91	3.87/4.48
8	8.95/8.01	9.18/7.79	4.75/6.47	4.67/4.16

*Each value represent plasma glucose concentration in mmol/l in male/female at Day 28

Urine analysis showed glycosuria in some animals at 200 U/kg/day, but not in recovery animals. Sponsor explains that this was due to high glucose levels in some biochemical investigations.

Ophthalmic Examinations: No drug related effects were observed.

Organ Weights: Analysis of organ weight data after 4 weeks of treatment showed a slightly lower absolute liver weight in the mid- and high-dose groups (control 18.2 g; with new X14 at Mid, 16.5 g; High, 15.9 g, and with old X14 High, 16.7 g), the relative liver weights were increased by up to 12%. The absolute ovary weight in the mid- and high-dose groups were increased (control 88.4 with new X14 at Mid, 95.2 mg; High, 112.6 mg, and with old X14 High, 94.2 mg), the relative ovary weights were increased by up to 28%. In recovery animals, the liver weights were slightly higher (19.5 and 18.2 with new and old X14 resp vs 17.4 in controls), however, the weight of ovaries remained significantly high (by 28%) in these animals (83.8, 82.9 vs 65.6 mg in controls). The absolute and relative organ weights of other organs were not remarkable different from those of controls.

Pathology: In thyroids, ectopic thymic tissue was noted in 1 and 3 of 10 males, and 2 and 2 of 10 females at high dose with both new and old X14 (vs none in controls). There were no other dosage or treatment related macroscopic or microscopic pathological changes.

Antibody Determination: Antibodies against X14 were found in all treated animals after 4 weeks. The frequency of antibody positive animals appeared to correlate to the given dose (5/10, 5/10, 20/20, and 20/20 animals had the presence of antibodies at 10, 50 and 200 U/kg/day of new X14, and 200 U/kg/day of old X14 resp) . The frequency of antibody positive animals was still high even after 4-week recovery period (9/10 animals had the antibodies with new and old X14), suggesting no difference in the antibody formation between old and new X14.

Toxicokinetics. Highest insulin X14 levels were noted at 1 and 5 hrs. These are shown in Table 7. No significant differences were noted with new vs old X14

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Table 7. Mean plasma levels of old and new X14 in 28-day subcutaneous toxicity study in rats (n=9/sex/dose), at doses of 10, 50, and 200 U/kg/day .

Dose U/kg/day	Day 1 (nM)		Day 28 (nM)	
	Mean plasma conc at 1 hr	Mean plasma conc at 5 hr	Mean plasma conc at 1 hr	Mean plasma conc at 5 hr
Males				
New X14-10	1.9	8.7	6.8	9.2
New X14-50	44.1	81.9	62.2	78.2
New X14-200	216.4	334.0	261	440
Old X14-200	226.3	296.9	229	314
Females				
New X14-10	2.8	0.7	4.4	3.9
New X14-50	38.4	31.5	49.2	74.0
New X14-200	202.7	189.6	199	269
Old X14-200	141.7	142.4	238	334

Conclusions: In a 4-week toxicity studies in rats with 4-week recovery period in rats with the new drug (X14 at doses of 10, 50 and 200 U/kg/day) and old drug X14 (200 U/kg/day), at high doses, ectopic thymic tissue was noted in 1 and 3 of 10 males, and 2 and 2 of 10 females in thyroids, with both new and old X14 (vs none in controls). Pharmacological response was associated with one instance of hypoglycemic death at the high dose. At 200 U/kg/day, increase in body weight and food consumption (by up to 13%) was observed. Antibodies (correlated with the dose) were found in all treated animals after 4 weeks treatment with X14 (new/old), as well as after drug free recovery periods. The tolerated dose of the drug in this (1-month) rat toxicity study was 50 U/kg/day.

One Year-Toxicity Study in Rats after Twice Daily Subcutaneous Injection, (Study No. 940301):

Sponsor's ID Study #: 940301

Amendment #, Vol #, and page #: Volume 19, page 1, Tk-volume 28, page 233

Conducting laboratory: _____

_____ Sponsor: Novo Nordisk A/S, Denmark.

Date of study initiation: November 16, 1994

GLP compliance: Yes

QA Report: Yes (X) No (), Is the evaluation based on a final, QA report: Yes

Methods: This study compared the effects of the new drug X14 (at 5, 25, and 100 U/kg/bid) vs with recombinant human insulin HM(ge) at 100 U/kg/bid in rats, for 52 weeks.

Dosing information:

species: Crl:CD BR rats

#/sex/group or time point: 32/sex/group

age: ~26-28 days old

weight: Males 79-91 g, females 72-85 g.

satellite groups used for toxicokinetics: 9/sex/group were used for TK

Dosage groups in administered units: Four groups (20 rats/sex/group) were given either vehicle (0.15% phenol, 0.172% M-cresol and 1.60 % glycerol) or X14 (an old process drug, i.e process A) twice daily by subcutaneous injections at doses of 5, 25, 100 U/kg/bid (or total dose of 10, 50, 200 U/kg/day) for 52 weeks. The fifth group received HM(ge) at twice daily dose of 100 U/kg/bid (or total dose of 200 U/kg/day). Additional 5 satellite groups (n=12/sex/group) received the drug similarly for TK studies, but since the animals were dying at 200 U/kg/day in group 4 and 5, satellite animals were also included in the main study and their doses were reduced. In week 25 (day 1), in groups 4 and 5, the drug was reduced by 50% to 50 U/kg/bid. In week 27 (day 4), all groups changed from twice to once daily dose, and for groups 4 and 5, the daily dose was 100 U/kg/day. In week 38 (day 1), in groups 4 and 5, the drug was further reduced to 75 U/kg/day (see appendix). Full necropsy and histology were performed on TK animals as well.

Route, form, volume, and infusion rate (if i.v.): Subcutaneous injections were given twice daily, 4 hrs apart, at a volume of 0.5 ml/kg, for up to weeks 25, and after that once daily for up to week 52.

Drug, lot #: X14 old process drug, Batch No. 06594 , 06894 , and 07094 HM(ge) Batch # 06194

Formulation/vehicle: Vehicle was 0.15% phenol, 0.172% M-cresol and 1.60 % glycerol

Times at which Observations are made:

Clinical signs: Daily

Body weights: At The time of allocation to groups, on the day of Tx and once a week thereafter.

Food consumption: Weekly.

Ophthalmoscopy: Before treatment and during weeks 13, 26, and 52 of treatment.

Hematology: Prior to the first dose and at the start of week 4

Clinical chemistry: Prior to the first dose of the morning during weeks 12, 25, and 51 of treatment

Urinalysis: Overnight urine samples were collected during week 12 and 25.

Gross pathology: At sacrifice at 52 weeks.

Organs weighed: These are highlighted in the histopathology table

Histopathology:* At sacrifice (from controls and HD and animals that died). Incidences of mammary tumors were analyzed. Testes and epididymides were stained with Periodic Acid Schiff (PAS) reagent. Liver sections were stained with Oil-O-Red O, and with PAS for glycogen. Also mammary gland (MG), lymph nodes from MG, and

subcutaneous masses were examined in all animals.

Toxicokinetics (in satellite animals): Days 1 and after weeks 13, 26 and 52 of treatment, at 0, 1, 3, 4, 5, and 8 hr. Plasma glucose levels were also measured in these animals.

Other (antibody determination in main study animals): Prior to first dose, and during weeks 12, 25 and 51.

Results:

Mortality: In all groups there were 32 rats/sex/dose, including 12 rats from satellite group. 18 of 32 male, and 18 of 32 female animals died in the high dose groups, thus mortality was 56% at a high dose (Table 8). In the mid dose group 25% of female rats died, while only 12% of male rats died as shown below. Sponsor indicates that total drug related deaths were 0, 1, 3, 17 and 20 in males, and 0, 1, 6, 15 and 15 in females at 0, 10, 50 and 200 U /kg/day of X14, and 200 U/kg/day of HM(ge) resp. Thus there were similar number of deaths with the drug X14, or with regular human insulin, at high dose. The animals were found dead prior to dosing at the morning check. Pathological findings showed hypoglycemia at the time of death or empty GI contents and haemorrhagic depression/erosions of the stomach wall.

Table 8. Mortality data in 1-year rat toxicity study:

Week	X14 Dosage(U/kg/day) in Male				X14 Dosage(U/kg/day) in Female			
	Control	10	50	200	Control	10	50	200
N	32	32	32	32	32	32	32	32
1-24	0	0	2	8	2	1	3	11
25-27	0	0	0	2	1	0	0	1
28-37	1	0	0	4	0	0	0	1
38-53	1	2	2	4	5	0	5	5
1-54	2	2	4	18	3	6	8	18
# Surv.	30	30	28	14	29	26	24	14
% Surv.	94	94	88	44	91	81	75	44

#Surv and % surv = Indicate the number and % of animals survived at the end of 52-week study. Due to the number of deaths in the high dose group, the dosing regime was amended as follows: Day 1 Week 25, the high dose reduced by 50%; Day 1 Week 38, the high dose reduced by 25% to 75 U/kg/day.

Clinical Signs: A total of 19 male and 23 female rats experienced apparent hypoglycemic episodes (between 2-4 hrs after dosing). Plasma glucose in these rats was < than 2 mmol/l, and administration of glucose did not prevent the subsequent death in most of these rats. Following changes in dose regimens in weeks 25, 27 or 38 did not improve the incidence of hypoglycemic episodes.

Body weight and Food Consumption: During the first 24 weeks of the study, there was an increase in body weight gain for rats of either sex receiving 50 and 200 U/kg/day. After the 50% reduction in dosage in the high dose group after 24 weeks of treatment, a transient weight loss was seen (up to 30%). However, the final reduction in X14 dose after 37 weeks of treatment showed no clear change in growth pattern, which indicates that there is no obvious relationship between the insulin dose and bodyweight changes. Food consumption for both sexes at the low and the mid dose groups was essentially similar to the controls, although the high dose increased the food consumption by 5-15% in female and 10 to 20% in male rats. Likewise, water consumption increased 10 to 20% in males only.

Ophthalmic Examination: There were no ocular changes considered related to treatment, although the number of survivors in the high dose group was rather small.

Hematology: In weeks 12 and 25 there was a slight (< 10%) reduction in red cell counts in males of the high dose group with the drug or HM(ge), which was not statistically significant. In contrast, MCH and MCHC were increased (by 5-9%) at high doses with both, the drug or HM(ge).

Biochemistry: Triglyceride (TG) increased at 200 U/kg/day, in both sexes in weeks 12, 25 and 51, values are shown at 51 weeks at 0, 10, 50, 200 U/kg/day with X14, and 200 U/kg/day with HM(ge) resp. Samples taken for clinical chemistry showed increased glucose levels in treated animals compared to controls, which is contrary to what is indicated under the mean plasma glucose concentrations below (which were decreased 1- hr after the drug dosing in a separate sampling). No explanation was provided by the sponsor for this discrepancy, except that the timing of blood sampling may have been different, and may explain it.

Parameter	males	females
glucose	124, 144, 176, 170, 185	132, 140, 139, 159, 164
TG	137, 164, 146, 194, 166	125, 106, 97, 147, 141
Urinary volume	5.9, 7.1, 8.7, 9.9, 10.5	7.1, 6.1, 8.7, 9.7, 6.6

Urine analysis showed increased urinary volume, along with decreases in total protein, and there was evidence of glycosuria in some animals at high doses of the drug or insulin, which may be due to high blood glucose levels.

Mean plasma glucose concentration: These were decreased in all treated groups to a similar level (4-5 mmol/l), one hour after the first dose on Day 1 and Weeks 13 and 26. Recovery by 4-5 hrs was initially observed, but in mid-high dose groups the plasma glucose remained low at all time points after the first few days. At Week 52, the glucose level at 1 hour post dose was slightly lower for all treated groups (males:3-4, females:2-4 mmol/l).

Organ Weights: At 200 U/kg/day, absolute weights of following organs were altered: